Environmental Health Perspectives
Vol. 97, pp. 153–157, 1992

Phagolysosomal pH and Dissolution of Cobalt Oxide Particles by Alveolar Macrophages

by Margot Lundborg,[1] Rolf Falk,[2] Anne Johansson,[1,3] Wolfgang Kreyling,[4] and Per Camner[1]

We studied phagolysosmal pH in rabbit alveolar macrophages (AM) incubated with 0–15 μM chloroquine. There was a dose-related increase in pH with chloroquine concentration. Electron microscopy showed that chloroquine increased lysosomal size. In a second experiment we studied dissolution of radiolabeled cobalt oxide particles by rabbit AM, phagolysosomal pH, and lysosomal size. The cells were incubated for 2 days with 0, 2.5, and 10 μM chloroquine. Size and pH increased with chloroquine concentration. Dissolution of cobalt particles by the AM did not clearly change with pH. In a third experiment, dissolution of cobalt oxide particles in 0.1 M acetate buffer in saline with pH 4.0, 5.0, and 6.0 was studied. At the same pH, dissolution in acetate buffer was faster than in the AM, and the dissolution appeared to decrease faster with increasing pH than in the AM. A simple model for dissolution of a particle in a phagolysosome was proposed. This model predicts the types of differences in dissolution between AM and buffered saline.

Introduction

The majority of insoluble particles deposited in the alveolar part of the human lung are mechanically cleared slowly with half-times up to several years (*1–4*). However, the particles are phagocytized by alveolar macrophages and these cells can efficiently dissolve many inorganic particles of materials that have low solubility in aqueous solution (*5–9*).

Phagolysosomal pH in alveolar macrophages from several species has been estimated to be around 5 (*10–13*). This low phagolysosomal pH enables the macrophages to dissolve metal particles. Alveolar macrophages dissolve lead arsenate particles at a faster rate, and arsenic trisulfite particles at a slower rate, than the incubation medium alone. In aqueous buffered solution, the solubility of lead arsenate particles increases and the solubility of arsenic trisulfide particles decreases with decreasing pH (*9*). At least some of the particles used in these experiments with macrophages, e. g., manganese dioxide and lead arsenate particles, show a marked increase in dissolution rate with decreasing pH in aqueous solution (*5*).

[1]Section of Lung Medicine, Unit of Environmental Hygiene, Institute of Environmental Medicine, Karolinska Institute, Stockholm, Sweden.
[2]Swedish Radiation Protection Institute, Stockholm, Sweden.
[3]Wenner-Gren Institute, University of Stockholm, Sweden.
[4]Gesellschaft für Strahlen- und Umweltforschung mbH, Project Inhalation, D-8042 Neuherberg, Federal Republic of Germany.
Address reprint requests to M. Lundborg, Section of Lung Medicine, Unit of Environmental Hygiene. Institute of Environmental Medicine, Karolinska Institute, Stockholm, Sweden.

We incubated macrophages with different concentrations of chloroquine, a weak base that can elevate lysosomal pH (*12*), and studied the phagolysosomal pH and ultrastructure of the macrophages. The dissolution of cobalt oxide particles by the macrophages was also investigated and compared with the dissolution in buffered solutions at various pH values. A simple model for dissolution of a particle in a phagolysosome is discussed.

Materials and Methods

Design

In the first experiment, we investigated the relationship between chloroquine concentration (0, 2.5, 5, 10, 15 μM) and phagolysosomal pH in the macrophages as well as the effect of chloroquine on the ultrastructure of the cells during 3 days of incubation. The macrophages were obtained by lavage from three rabbits.

In the second experiement, dissolution of uniform cobalt oxide particles by macrophages, phagolysosomal pH, and the size of lysosomes were studied in macrophages incubated for 2 days with 0, 2.5, and 10 μM chloroquine. Macrophages were obtained by lavage from six rabbits.

In the third experiment, we studied the dissolution of the cobalt oxide particles in buffered solutions (0.1 M acetate buffer in saline) with pH 4.0, 5.0, and 6.0.

Macrophages

Lungs from healthy New Zealand white rabbits, 2.8–3.2 kg, were dissected and lavaged with Hank's balanced salt solution

(without Ca^{2+} and Mg^{2+}, pH 7.4, 37°C) (*5*). The lavage fluid was centrifuged at 300*g* for 10 min at room temperature and resuspended in complete medium (HEPES buffered medium 199, pH 7.4 with 15% rabbit unheated serum, penicillin 100 units/mL and streptomycin 100 μg/mL). The number of macrophages was estimated in a Bürker chamber.

In the studies of phagolysosomal pH, the number of cells/sample was 0.5×10^6 cells. In the dissolution studies and in the electron microscopic studies, the number/sample was 1.0×10^6 cells. The cell samples, in 2 mL complete medium, were added to coverslips in tissue culture dishes (Costar) (dissolution and pH studies) or Leighton tubes (electron microscopic study).

After 1 hr incubation (37°C with 5% CO_2 in air and 80% relative humidity), the cell medium was replaced with fresh medium with chloroquine and cobalt oxide particles (dissolution and electron microscopic studies) or chloroquine and silica particles (pH study).

Phagolysosomal pH

We studied phagolysosomal pH by using fluorescein-labeled amorphous silica particles (FSP) with mean diameter 3.0 ± 0.5 μm (± SD) as probes (*15*). Fluorescence intensities from individual FSP in phagolysosomes were measured using a technique for microscope fluorimetry (*16*). Two excitation filters were used; the first one had a transmission maximum at 452 nm and the second one at 489 nm. The half-maximum bandwidth was 8 nm for both excitation wavelengths. Fluorescent light above 515 nm was selected by a barrier filter.

For each time, we studied macrophages within a 2- or 3-day period, determined a standard curve between the ratio of fluorescence above 515 nm with the excitation wavelength 489 and 452 nm, and determined pH. Mixtures of 1.0 M citric acid/sodium citrate were diluted with four parts of Hank's balanced salt solution to give buffer solutions in the range pH 4–7 (*16*).

Dissolution of Cobalt Oxide Particles

To each macrophage sample and each control sample consisting of media without macrophages, 2.4×10^6 uniform porous ^{57}Co-labeled Co_3O_4 particles, 0.6 μm in diameter, were added (*17*). To each of the macrophage samples incubated for 0 or 2 days with chloroquine concentrations of 0, 2.5, or 10 μM, control samples without macrophages were also tested. The day 0 samples give information on the readily dissolved cobalt fraction on the particle surface and the cobalt fraction dissolved during the treatment after the incubation. In addition, parallel to the samples from five of the six rabbits, dissolution of duplicate samples of the test particles in 2 mL 0.1 M acetate buffer in saline of pH 5.0 was tested.

After incubation, we removed and collected the medium. Macrophages were removed from the coverslips with a rubber scraper. The medium and the macrophage suspension were mixed and centrifuged for 10 min at 350*g*. One percent Triton X-100 solution (0.5 mL) was added to the cell pellet and the suspension sonicated for 30 min to desintegrate the cells. We performed the same procedure with the control sample without macrophages. The residue and supernatant were then mixed and filtered through a 0.22-μm Millipore filter (type GS), and the radioactivity on the filter and filtrate were measured. The ^{57}Co activity on the filter was assumed to represent the amount of cobalt in particulate form, and the activity in the filtrate was assumed to represent the cobalt in soluble form.

In the third experiement, the same number of cobalt oxide particles as in the experiment with the macrophages above was added to 2-mL samples with 0.1 M acetate buffer with pH 4.0, 5.0, and 6.0. After 2 days at 37°C, the samples were filtered and the radioactivity in filter and filtrate measured.

Radioactivity Measurements

A high-purity germanium detector with a relative efficiency of 30% was used for all measurements. The gamma detector was housed inside a thick (10 cm) lead shield to obtain a low and stable background. The duration of each measurement was selected to give less than 3% statistical counting error. All measurements and calibrations were performed in the Environmental Laboratory at the Swedish Radiation Protection Institute.

Electron Microscopy

Macrophages were fixed and processed for electron microscopy (*15*). We estimated the volume density of the lysosomal compartment from sections of five randomly selected macrophages from each sample. We measured the area of the lysosomes in a standardized area of the macrophages with a digitizer (HIPAD) connected to a computer, and the quotient $A_{\text{lysosome area}}/A_{\text{macrophage area}}$ was estimated. Relative lysosome circumference was calculated from the perimeters of the lysosomes in the standardized area. the average circumference of the lysosomes in macrophages not exposed to chloroquine was normalized to 1.

Results

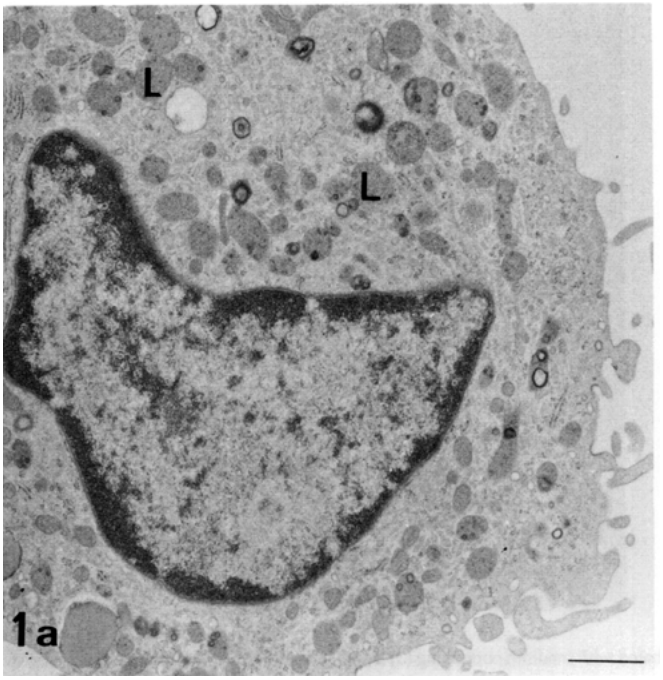
Table 1 shows phagolysosomal pH in macrophages exposed to different concentrations of chloroquine for 1 and 3 days. The mean values of phagolysomal pH were related to the concentration of chloroquine. Electron microscopy showed that lysosomes increased in size in all macrophage samples incubated with chloroquine (Fig. 1). Large vacuoles were seen in cells incubated with 5 μM or higher chloroquine concnetration.

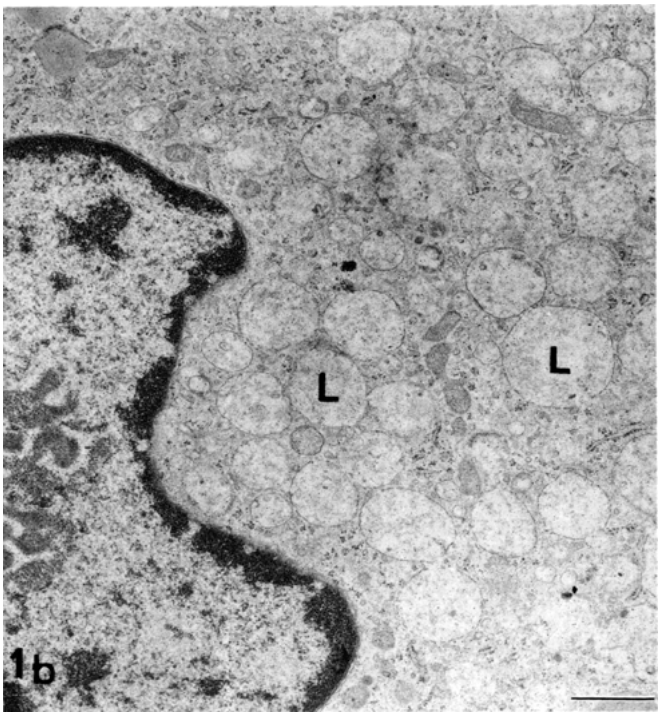
Table 2 shows circumference of lysosomes, relative volume of lysosomes in macrophages, phagolysosomal pH, and dissolution of cobalt oxide particles by macropahges and in controls without macrophages. There were dose-related increases in the phagolysosomal pH and in the size of the lysosomes with increased chloroquine concentration. For the 0 and 2.5 μM chloroquine concentrations, significantly more cobalt was dissolved by the macrophages than in the respective control samples ($p < 0.01$,

Table 1. Phagolysosomal pH in macrophage samples with different chloroquine concentrations, incubated for 1 and 3 days.[a]

	Chloroquine concentration, μM				
Day	0	2.5	5.0	10.0	15.0
1	5.1 ± 0.1	5.4 ± 0.02	5.5 ± 0.1	5.6 ± 0.1	5.7 ± 0.2
3	5.2 ± 0.1	5.3 ± 0.1	5.5 ± 0.1	5.6 ± 0.2	5.7 ± 0.2

[a]Data are given as means ± SD, $n = 3$.





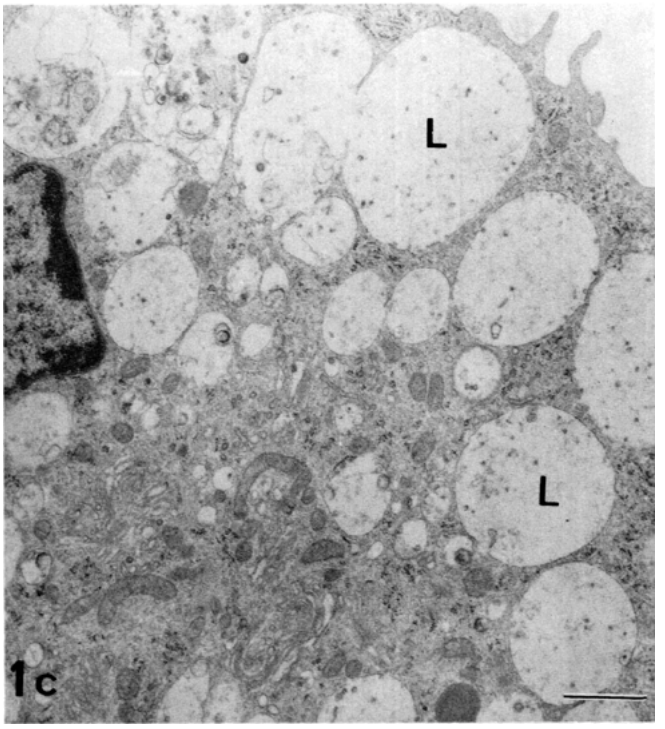


FIGURE 1. Lysosomes in rabbit alveolar macrophages, incubated with medium containing (*a*) 0 μM, (*b*) 2.5 μM, and (*c*) 10 μM chloroquine. Bar = 1 μM.

paired *t*-test), but not for the 10 μM concentration ($p < 0.05$). Figure 2 shows cobalt oxide particles in lysosomes of rabbit alveolar macrophages incubated with medium containing 0 μM and 10 μM chloroquine. Dissolution in 0.1 M acetate buffer in saline at pH 5 was similar in the duplicate samples, but there were rather large differences in dissolution among samples of macrophages from different rabbits tested in parallel (Table 3).

Table 2. Ultrastructural changes, phagolysosomal pH, and dissolved cobalt after incubation for 2 days with different chloroquine concentrations.[a]

	Chloroquine concentration, μM			
	Day 0	Day 2		
Parameter	0	0	2.5	10.0
Relative lysosome circumference[b]	1.0 ± 0.1	0.8 ± 0.1	1.2 ± 0.1	1.3 ± 0.2
Volume density of lysosomes in macrophages, %	15 ± 3	10 ± 3	25 ± 4	31 ± 6
Phagolysosomal pH	—	5.1 ± 0.2	5.4 ± 0.3	5.6 ± 0.3
Dissolved Co by macrophages, %[c]	—	1.8 ± 0.3	2.3 ± 1.2	1.6 ± 1.1
Dissolved Co in medium, %[c]	—	0.4 ± 0.3	0.7 ± 0.9	0.6 ± 0.8

[a] Data are given as means ± SD, $n = 6$.
[b] The mean of lysosome circumferences at 0 μM concentration at day 0 was standardized to 1.0.
[c] The values at day 0 were subtracted from the values at day 2. The mean of the day 0 values was 0.7; range 0.3–1.0%.

Dissolution in the acetate-buffered saline with pH 5 was significantly higher than that by the macrophages without chloroquine ($p < 0.01$, *t*-test). There was no clear decrease in dissolution by the macrophages with increasing pH (Fig. 3; Table 2).

Figure 3 shows the dissolution of the cobalt oxide particles in 0.1 M acetate buffer in saline with pH 4.0, 5.0, and 6.0. More cobalt was dissolved in the buffer than in the macrophages at comparable pH, and the decrease in dissolved material with increased pH was pronounced in the buffer but not in the macrophages.

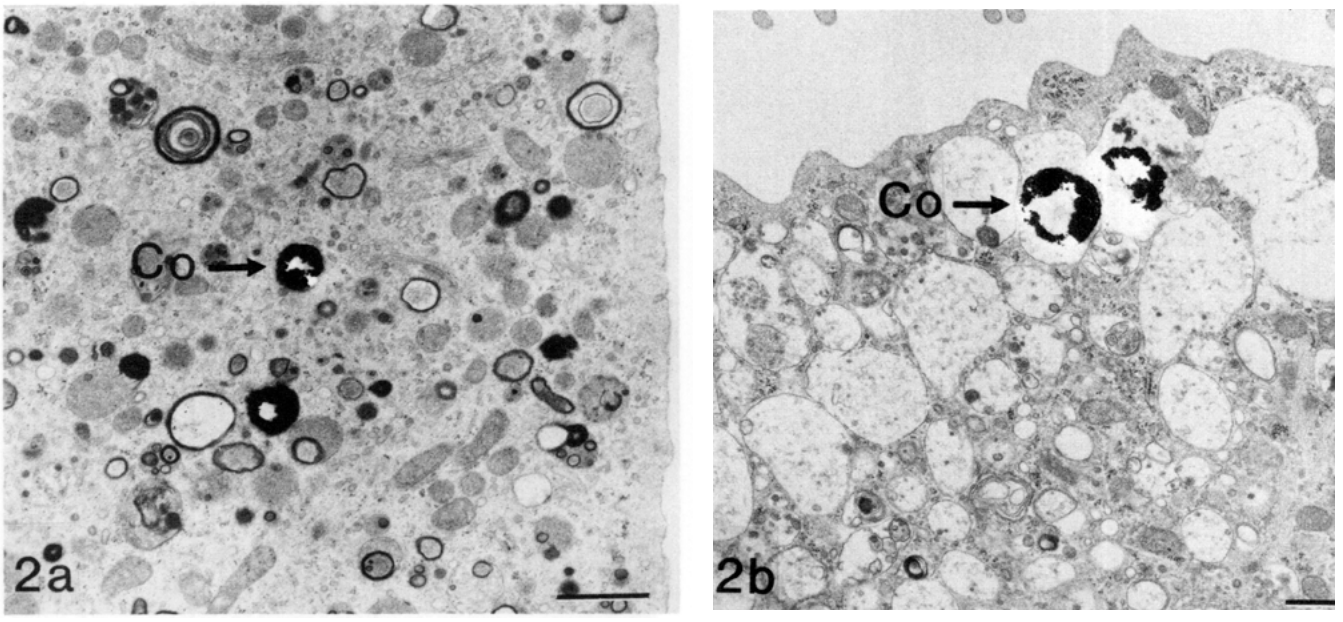


FIGURE 2. Cobalt oxide particles in lysosomes of rabbit alveolar macrophages incubated with medium containing (*a*) 0 μM and (*b*) 10 μM chloroquine. Bar = 1 μM.

Table 3. Dissolution of cobalt oxide particles in 0.1 M acetate buffer in saline at pH 5.0 during 2 days, duplicate samples a and b.

	% Dissolved	
Sample	a	b
1	3.3	3.5
2	5.7	5.9
3	6.3	5.9
4	7.7	7.4
5	6.8	7.8

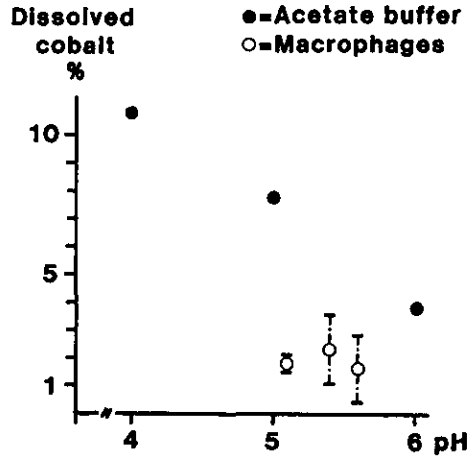


FIGURE 3. Dissolved cobalt in 2 days in acetate buffer in saline with pH 4, 5, and 6 and in macrophages with different pH.

Discussion

Chloroquine induced clear dose-related increases of phagolysosomal pH and size of the lysosomes. When the dissolution was related to the average pH at each chloroquine concentration dissolution, there was no clear decrease with increasing pH. The dissolution in the macrophages was clearly less than in saline with acetate buffer. This might be due to chelating capacity of the acetate buffer (*18*). However, our preliminary data indicate that there is no major difference in dissolution of the cobalt oxide particles in 0.1 M and 0.001 M acetate buffer (unpublished data) which still might be a high concentration compared with chelator concentrations in macrophages. The decrease in dissolution with increasing pH appeared to be less in macrophages than in saline.

A simple model for dissolution of a particle in a phagolysosome is a particle in a liquid-filled vesicle with fixed pH in a large volume of liquid. The dissolution rate of the particle material is proportional to the particle surface area and is dependent on the concentration of dissolved material from the particle in the vesicle; the higher the concentration, the lower the dissolution rate. Dissolved material leaves the vesicle through its membrane. The amount leaving per unit time is proportional to the area of the membrane and is dependent on the concentration in the vesicle: i.e., the higher the concentration, the more material leaves. A steady-state situation between material dissolving from the particle and material leaving the vesicle is assumed.

In this model the dissolution rate of a metal particle should be lower inside the vesicle than if it is directly placed in the large liquid volume. The difference may of course be small if the transport rate through the membrane is close to that through the liquid layer of the same thickness as the membrane. The dissolution rate for a particle in a larger vesicle is faster than in a smaller vesicle because the steady-state concentration is lower in the larger than in the smaller one. The model thus predicts the type of differences between our dissolution experiments with macrophages and those in buffered saline: *a*) lower dissolution rate in macrophages than in saline with the same pH and *b*) a de-

creased relationship between pH and dissolution rate because the effect of the increased pH is compensated for by an increase in phagolysosomal size.

The reasons above suggest that it would be of value to develop a more detailed model for dissolution rates of particles in phagolysosomes of various sizes. Particle size itself affects the size of the phgolysosome. Particles of the same size but of different material may affect size, e.g., there is often vacuolization around yeast particles but not silica particles (*15*). Also, inhalation of pollutants may affect size, e.g., trivalent chromium in soluble form induces large lysosomes in alveolar macrophages (*19*).

This study was supported by the research fund of the National (Swedish) Environmental Protection Board.

REFERENCES

1. Bailey, M. R., Fry, F. A., and James, A. C. The long-term clearance kinetics of insoluble particles from the human lung. Ann. Occup. Hyg. 26: 273–290 (1982).
2. Bailey, M. R., Kreyling, W. G., Andre, B., Batcholar, A., Collier, C. G., Drosselmeyer, E., Ferron, G. A., Foster, P., Haider, B., Hodgson, A., Masse, R., Metivier, H., Morgan, A., Müller, H., Patrick, G., Pearman, I., Pickering, S., Ramsden, D., Stirling, C., and Talbot, R. J. An interspecies comparison of the lung clearance of inhaled monodisperse cobalt oxide particles. Part 1: Objectives and summary of results. J. Aerosol Sci. 30: 169–188 (1989).
3. Bohning, D. E.. Atkins, H. L., and Cohn, S. H. Long-term particle clearance in man: normal and impaired. Ann. Occup. Hyg. 26: 259–271 (1982).
4. Philipson, K., Falk R., and Camner, P. Long-term lung clearance in humans studied with Teflon particles labeled with ^{51}Cr. Exp. Lung Res. 9: 31–42 (1985).
5. Lundborg, M., Lind, B., and Camner, P. Ability of rabbit alveolar macrophages to dissolve metals. Exp. Lung Res. 7: 11–22 (1984).
6. Lundborg, M., Eklund, A., Lind, B., and Camner, P. Dissolution of metals by human and rabbit alveolar macrophages. Br. J. Ind. Med. 42: 642–645 (1985).
7. Kreyling, W. G., Ferron, G. A., Godleski, J. J., Haider, B., and Kariya, S. T. The dissolution of monodisperse, porous cobaltosic oxide particles in the dog's lungs and in its alveolar macrophages. In: Aerosols: Formation and Reactivity (W. Schikarski, H. J. Fissan, and S. K. Friedlander, Eds.), Pergamon Press, Oxford, 1986, pp. 232–239.
8. Andre, S., Metivier, H., Lenfenois, G., Boyer, M., Nolibe, D., and Masse, R. Beryllium metal solubility in the lung. Comparison of metal and hot-pressed, forms by *in vivo* and *in vitro* dissolution bioassays. Hum. Toxicol. 6: 233–240 (1987).
9. Marafante, E., Lundborg, M., Vahter, M., and Camner, P. Dissolution of two arsenic compounds by alveolar macrophages. Fundam. Appl. Toxicol. 8: 382–389 (1987).
10. Jensen, M. S., and Bainton, D. F. Temporal changes in pH within the phagocytic vacuole of the polymorphonuclear neutrophilic leukocyte. J. Cell Biol. 56: 379–388 (1973).
11. Jacques, Y. V., and Bainton, D. F. Changes in pH within the phagocytic vacuoles of human neutrophils and monocytes. Lab. Invest. 39: 179–185 (1978).
12. Ohkuma, S., and Poole, B. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. U.S.A. 75: 3327–3333 (1978).
13. Geisow, M. J., D'Arcy Hart, P., and Young, M. R. Temporal changes of lysosomes and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy. J. Cell Biol. 89: 645–652 (1981).
14. Nilsen, A., Nyberg, K., and Camner, P. Intraphagosomal pH in alveolar macrophages after phagocytosis *in vivo* and in vitro of fluorescein-labelled yeast particles. Exp. Lung Res. 14: 197–207 (1988).
15. Nyberg, K., Johansson, A., and Camner, P. Intraphagosomal pH in alveolar macrophages studied with fluorescein-labeled amorphous silica particles. Exp. Lung Res. 15: 49–62 (1989).
16. Nyberg, K., Johansson, U., Rundquist, J., and Camner, P. Estimation of pH in individual alveolar macrophage phagolysosomes. Exp. Lung Res. 15: 499–510 (1989).
17. Kreyling, W. G., and Ferron, G. A. Production of cobalt oxide aerosols with a modified spinning top aerosol generator. J. Aerosol. Sci. 15: 367–371 (1984).
18. Kreyling, W. G., Neuner, M., and Matejkova, E. Analysis of intraphagolysosomal dissolution of test particles in canine alveolar macrophages. J. Aerosol. Sci. 19: 1071–1074 (1988).
19. Johansson, A., Robertson, B., Curstedt, T., and Camner, P. Alveolar macrophages abnormalities in rabbits exposed to low concentrations of trivalent chromium. Environ. Res. 44: 279–293 (1987).